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Advanced method of secondary metabolite extraction and quality analysis

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Abstract
Plant had been used as medicine in ancient. Now day's Pharmaceutical companies start processing of medicinal and aromatic plants in their formulation by using extraction of active components. Extraction of plant components likes volatile, Essential or ethereal oils and mixtures composed of volatile liquid and solid compounds depend upon their composition and their boiling point. Now days there are several processes like distillation, enfleurage, maceration, expression, solvent extraction and fluid extraction are available for extraction of plant component and different kinds of laboratory facilities are needed for the extraction and quali-quantitative analysis. Recent extraction technology such as Accelerated Solvent Extraction or Microwave Assisted Extraction in combination with hyphenated techniques such as Gas Chromathography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) represent a modern approach to perform fast and reproducible analytical methods for the quality control of secondary metabolite production from plant material.

Keywords: Secondary metabolite, phytoconstituents, chromatography, maceration

Introduction
Researchers from a variety of scientific disciplines are confronted with the challenges of extracting the biological material with solvents, often as a first step towards isolating and identifying the compounds responsible for biological activities associated with the biological materials and its extracts. Although chemistry has provided human kind with a large number of different materials to make life easier, but we still cannot make use of some of the resources effectively as mother earth does. Plants and their biological sources produce and supplies massive natural products called metabolites which are not directly involved in growth and reproduction but their existence can be demonstrated biochemically, genetically and physiologically. The function of these so called secondary metabolites is often closely related with the functional groups present in their structure. Thus, natural metabolites are suppressants, insecticides, fungicides, herbicides as well as oils, aromas, fragrances originated from natural sources. IPM programmes have demonstrated that current levels of pesticide use in manycircumstances are not necessary and, frequently, are even counter-productive. Excessive andotherwise in appropriation of pesticide is an unnecessary burden on farmers' health andincome, on public health, and on the environment (Mukhrejee, 2002). Biorational insecticideshave emerged as an alternative or as supplemental forms for pest control. The use ofbiopesticides will help in preventing the discarding of thousands of tons of pesticides on theearth and provide the residue free food and a safe environment to live.
Further interest arises from growing awareness about secondary metabolites of organisms and plants, which serve biologically and ecologically significant as defensive compound and chemical messenger. The isolation of metabolite from natural sources soon discover the need of sizeable laboratories in the apparently regular sample preparation steps that convert crude material in to extract that can be analyzed chemically and biologically.
Extraction process for metabolites can be depends on the partition of components between solvent phases and formation of solid residual, solvent volume concentration should be gradient between micelle and finally it has to become zero to attain equilibrium stage. The equilibrium state depends on property and nature of metabolite, quantity, solvent selection, mixing ratio, temperature, p^H and method of preparation of solvent.

Characters of phytoconstituents
The basic knowledge about the phytoconsttuents nature and their characteristics is essential to select the method and solvent for extraction. Nature of phytoconstituents involves p^H, polarity, thermo stability etc.

pH

The extraction of the component is extensively affected by the pH of solvents. Non- polar alkaloids can't be extracted into aqueous system but can be extracted into polar aqueous acid due to their basic nature and salt formation in acid. Fatty acids, phenols and other acidic phytochemicals are extracted using solvents at alkaline pH. The compounds should not break down at employed pH values, e.g. esters are prone to hydrolysis in alkali and glycosides lose the sugar moiety in acid. (Kokate, 2009) [40].

Polarity

As a quote "like dissolves like". Polar compounds dissolves in polar and non polar dissolves in non polar solvents respectively. Solvent selection depends on either nature of phytoconstituents directly or extraction of component followed by removal of interference first (example for curcumin extraction defatting is done first and then proceeded for extraction by methanol and chloroform). It is rudimentary to study about the relationship between the extraction method applied and the physicochemical properties of the substances to be extracted. (Sethi & Sethi, 2001) [62-63].

Thermo stability

Normally solubility of component increases with raise in temperature. Higher temperatures facilitate penetration of the solvent into the cellular structures of herbs. Thermo labile components are sensitive to higher temperatures. The artifacts may arise in presence of solvent/components on heating which may degrade the biologically active compound or may exert toxicity or may create separation problem. (Stahl Egon, 2005) [65].

Choice of Solvents

Secondary metabolites have different degrees of polarity so the solvent(s) should be chosen for the extraction should be considered carefully to ensure dissolution of secondary metabolites under study.

Solvent should have following properties:

1. Easy to remove
2. Inert
3. Nontoxic
4. Not easily inflammable
5. No interaction or less chemical interaction

Solvent (mixture of solvent) is employed to dissolve the secondary metabolite and finally to diffuse out the dissolved solute into bulk solvent phase.

Solvent employed are

1. Polar: Water
2. Non-polar: Petroleum ether, chloroform, Diethyl ether
3. Semipolar: Ethanol, Acetone
4. Azetropic mixtures

Polar Solvents

The polar compounds like polysaccharides, phenols, aldehydes, ketones, amines, and other oxygen containing compounds dissolve in water due to formation of hydrogen bonding. The solubility of aliphatic alcohol increases the solubility of the compound in water decreases. Additional polar groups are present in the molecule, as found in propylene glycol, glycerin, and tartaric acid, water solubility increases greatly due to addition of polar groups. Branching of the carbon chain reduces the nonpolar effect and leads to increased water solubility (tertiary butyl alcohol is miscible in all proportions with water, whereas n-butyl alcohol dissolves to the extent of about 8 g/100 ml of water at 20°C).

The polar solvents such as water act as solvents according to the following mechanisms

1. Normally polar solvents have high dielectric constant which reduces the force of attraction between oppositely charged ions in crystals such as sodium chloride or molecule. Polar solvent like water has a dielectric constant of 80 while which dissolve polar component rapidly than non-polar solvent chloroform, which has a dielectric constant of 5 and due to low dielectric constant, ionic compounds are practically insoluble in non-polar organic solvents.
2. Polar solvents break covalent bonds of potentially strong electrolytes by acid-base reactions since these solvents are amphiprotic. For example, water brings about the ionization of HCI as follows:
   a) Weak organic acids are not ionized appreciably by water
   b) Their partial solubility is attributed instead to the hydrogen bond formation by with water. Phenols and carboxylic acids, however, are readily dissolved in solutions of strong bases.
3. Polar solvents has property of dipole interaction forces, particularly hydrogen-bond formation dut to which solvating molecules and ions become soluble and which leads to the solubility of the compound. The solubility of sodium salt of oleic acid and water is due to ion-dipole interaction. (Sethi, 2001) [62-63].

Non-polar Solvents

Non-polar solvents have low dielectric constants and dissolve non-polar solutes with similar internal pressures through induced dipole interactions. Ionic and polar solutes are insoluble or slightly soluble in non-polar solvents. Weak Van-Der-Waals and London type of forces are responsible for the solubility of molecules.

Semi-polar Solvents

Semi-polar solvents like ketones and alcohols can induce a certain degree of polarity in non-polar solvent like benzene is readily polarizable, becomes soluble in alcohol. Semi-polar compounds act as intermediate solvents which bring about miscibility of polar and non-polar liquids.

Azeotropic Mixtures

Azeotropes are mixture of different solvent with varying polarity which has near boiling points. They affect the dissolution properties and degree of extraction of extractable matters. To utilize this phenomenon fully it is recommended that the composition of the menstrum be chosen so that a binary or ternary azeotropic mixture is produced. This has the advantage that upon concentration of the extracts, the solvent boils constantly and the condensate, perhaps after a small correction by replacement of components preferentially retained in the drug residue, can be reused. Azeotropic mixtures have great potential to extract active phytochemical metabolites from the crude drugs depending on their varied chemical nature as they can extract large numbers of constituents based on its nature.

Influence of Solvents

A component may behave like strong electrolyte or non-electrolyte, depending on pH of solution. Precipitation of components occurs, when the pH of solution is adjusted to such a value at which un- ionized molecules are produced in sufficient concentration to exceed its solubility.

Solvent-Solute interactions

Polar solvents like water is a good solvent for salts, sugars etc while non-polar solvents like mineral oil and benzene are often solvents for substances that are normally only slightly soluble in water. It proves the doctorine of “like dissolves like". (Sathl eagon, 2005)

Combined Effect of pH and Solvents

The solvent affects the solubility of a weak electrolyte in a buffered solution in two ways,

i) The addition of alcohol to a buffered aqueous solution of a weak electrolyte increases the solubility of the un-ionized species by adjusting the polarity of the solvent to a more favourable value.
ii) Being less polar than water, alcohol decreases the dissociation of a weak electrolyte, and the solubility of the drug goes down as the dissociation constant is decreased (pKa is increased). (Colegate*et. al.*2008)

Solvents, Problems and Limitations

The secondary metabolites must dissolve in solvent chosen for the extraction. The solvent chosen for the extraction must have following qualities

1. It must dissolve the secondary metabolites
2. It should be easy to remove.
3. It should be inert.
4. It should be nontoxic.
5. It should not be easily flammable.
6. It should not form any type of unstable substance during extraction or mixing.
7. Solvents should be distilled or even double distilled prior to use if they are of low or unknown quality.
8. Solvent must be free from plasticizers like dialkyl phthalate, tri-n-butyl acetyl citrate and tri-butyl phosphate which are commonly found as impurities in solvents and mat impart stability problem. (Kokate *et al*., 2009, Buchanan *et al*., 2007) [40, 12].

Extraction Strategy

Isolation of compound

The 3 stages are basic

1. First step of extraction involves solvent penetration into herb cells/tissues, solubilization of secondary metabolites and finally release the dissolved secondary metabolites in solvent of extraction. Solvents of varying polarity are used alone or in combinations for extraction depend on component. So a large proportion of the unwanted material is removed. (Maceration, Digestion, Decoction, Soxhlet extraction, Supercritical chromatography, Hydro-distillation, Enflurage, Eculle, Supercritical fluid chromatography)
2. Second step is fractionation with subsequent analysis: Either open silica column or counter current distribution/liquid-liquid extraction is used to separate or fractionation of components. (Distillation, Sublimation, Evaporation, Fractional crystallization, fractional distillation, Sublimation, Fractional crystallization, fractional distillation, GC, CCD).
3. The third final stage is achieved by HPLC or TLC which involves separation of desired component in adequate purity (TLC, GC, GLC, Mass, NMR, UV, Fluorimerty)

Extraction is followed either by

1. Powdered dried material is directly extracted to achieve extract
2. Or first defeat the material and then extraction of desired component
3. Or fresh plants (e.g. leaves) can be homogenized or macerated with alcohol

Selection of extraction method depends on

1. Nature of component
2. Nature of material to be used
3. Solvent system available

Techniques used to enhance extraction

1. Ultrasound may enhance the extraction process for some plant materials, eg., the preparation of a 50% ethanolic solution of opium for the assay of alkaloid.
2. Use of microwaves can also enhance extraction.
3. By altering pH
4. By stirring
5. By reducing the particle size
6. By changing the polarity of solvents

Procedures for Extraction of Herbal metabolites

During the extraction of the herbal metabolites the rinsing of extractive substances out of disintegrated plant cells, swelling of the drug plant material in order to increase the permeability of the cell walls, Penetration of the solvent into the plant cells and swelling of the cells, Dissolution of the extractive substances, Diffusion of the dissolved extractive substances out of the plant cell and finally the dissolution of extractive substances out of intact plant cells by diffusion take place. It has been found that the liquorice root when extracted with the solvent (0.25% ammonia solution) it penetrates into the roots more rapidly and this process is accelerated by raising the temperature. The steeping and swelling process is strongly influenced by particle size and is more evident radically. Upon penetration into the plant material, the solvent becomes enriched with extractive substances and hence the highest content of extractive solvent front. Some general points should be considered in terms of the extraction process, such as the overall characteristics of the secondary metabolites to be extracted (e.g. some glycosides are thermolabile or pH-sensitive). Although the normal practice is 'to apply technique to obtain a crude extract from a plant material, e.g. an acid-base shakeout to prepare an alkaloidal extract, because of the structural diversity within a given natural product group and their possible special requirements, it is advisable to consult specific reviews, papers, and books in order to prevent the avoidable loss of desired bioactive metabolites caused by the use of an inappropriate extraction technique. The simplest extraction processes employed may be classified as follows: extraction with organic solvents: percolation, maceration, and extraction using a Soxhlet apparatus; and extraction with water: infusion, decoction, and steam distillation. The most popular method of extraction is to use a liquid solvent at atmospheric pressure, possibly with the application of heat. Other methods include steam distillation, supercritical fluid extraction and the use of liquefied gases under moderate pressure. The choice of method depends on the factors listed

above as well as the intrinsic advantages and disadvantages of the procedures. (Rahman, 1989) [55].

Supercritical fluid extraction

The process of separation of one component which is extracting from matrix by using supercritical fluid is known as supercritical fluid separation. Supercritical fluids showed property intermediate between those of the liquid and gaseous phases, for any substance it is a condition above the critical temperature and pressure. SFE offers many advantages as follows:

1. It leads to lower solvent usage
2. Controllable selectivity
3. Cleaner extracts and less thermal degradation as compared to conventional solvent extraction and steam distillation methods,

Super critical carbon dioxide (SCO2) - with its particularly attractive properties such as non-toxicity, non-flammability, non-corrosiveness, chemical inertness, low critical temperature (304"K), moderately low critical pressure (73 atm), easy availability, co-effectiveness and environmental acceptability is the preferred solvent for many super critical extractions. Liquid carbon dioxide is completely miscible with components of essential oils like aldehyde, ketones, esters and alcohols. At same time, proteins, starches, mineral salts and water are insoluble in liquid carbon dioxide. Essential oils obtained by liquid carbon dioxide extraction are superior to that obtained through steam distillation and solvent extraction. Extraction of several natural products such as pyrethrins from chrysanthemum flower, essential oils from anise, caraway, clove, star anise, cinnamon and ginger are increasingly done by this process. (Rastogi *et al*., 1999, Stahl 2005) [65, 56-57].

Solid phase extraction

Solid phase extraction is process of separation of dissolve and suspended component from liquid mixture by using another component in the mixture according to their physical and chemical property.

Distillation

Distillation may be defined as separation of components of a mixture of two or more liquids by virtue of difference in their vapor pressure. There are three systems of distillation-

- Hydro distillation
- Hydro-steam distillation
- Steam distillation

Hydro distillation

Hydro distillation is the oldest method being used for separation of essential oil. In this method plant material is contact with boiling water in a crude metallic distillation unit. This process use principle of osmotic press principle of osmotic pressure to diffuse oil from the oil glands. The essential oil of a plant consists of many compounds which generally boil between 150° to 300° C. The vapors pass through a coiled tube contained in a water bath and condensate is obtained at the bottom of the condenser tube. The disadvantages are that the heat is difficult to control and hence the rate of distillation is variable. Also the possibility exists for local overheating and "burning" of the charge which can lead to poorer quality oil.

Hydro-steam distillation

To overcome the drawback of water distillation, modifications in techniques was developed. In this technique plant material is supported on a perforated grid or screen inserted at some distance above bottom of still. Water filled below the grid is heated which produce saturated and wet steam; produced steam pass through plant material and vaporized essential oil.

Steam distillation

A process of extracting essential oils from plant products through a heating and evaporation process is known as steam distillation. Steam distillation is a popular method for the extraction of volatile oils (essential oils) from plant material. This can be carried out in a number of ways. One method is to mix the plant material with water and to heat to boiling (distillation with water). The vapors are collected and allowed to condense, and the oil separated from the water. It resembles hydro-steam distillation except that no water is kept in bottom of still. This method is efficient and gives higher yields. However, it is not generally employed to delicate flowers. To maximize the yields of the oils, precautions must be taken to ensure efficient condensation of the steam and vaporized oil and collection of the condensate in such a way as to prevent loss of the volatile material. However, to avoid risk of explosion, a completely closed system must not be used. The advantages of this type of "dry" steam distillation are that it is relatively rapid, therefore charging and emptying the still is much faster and energy consumption is lower.

Maceration (Extraction with hot fat)

Maceration is process of extraction with hot oil or fat. In maceration, oil cells of fragrant flowers are ruptured by immersion in a hot fat or oil at 60-70°C which in turn absorbs essential oils. Fat is separated from spent flowers and reused for absorbing fragrance from next batch of fresh flowers. Fat retained by flowers is recovered by hydraulic pressing. Resultant perfumed pomade is frequently marketed as such but is often extracted with strong alcohol to yield extracts. This is very much the same technique used in solvent extractions, where solvents are used instead of the hot oil as used in maceration. (Rastogi *et al* 1999) [56-57].

Enfleurage (Extraction with cold fat)

Enfleurage is the process of extraction of fragrance by absorbing it from flowers in contact with cold fats. This process is adopted for fragrant flowers of jasmine and tuberose, which continue to manifest their characteristic fragrance even in plucked condition. Fats should be saturated and odorless to prevent entrance of fat odors. Refined lard or beef suet are preferred. Fat is thinly layered on both sides of a glass plate supported on a rectangular wooden frame or chassis. Fresh fragrant flowers are lightly layered on fat coated chassis.

Enfleurage gives a much greater yield of flower oil than other methods. Despite this advantage, enfleurage has lately been replaced by extraction with volatile solvents because enfleurage is a very delicate and lengthy process requiring much experience and labour.

Extraction with volatile solvents

Principle of extraction with volatile solvents is simple. Fresh flowers are charged into specially constructed extractors and extracted systematically at room temperature, with a carefully

purified solvent usually petroleum ether. Solvent penetrates flowers and dissolve natural flower perfume together with some waxes and albumins and colouring matter. Solution is subsequently pumped into an evaporator and concentrated at a low temperature. After the solvent is completely driven off in vacuum, concentrated flower oil is obtained. Thus, temperature applied during entire process is kept at a minimum; live steam as in case of distillation, does not exert its action upon delicate constituents of flower oil. Compared with distilled oils, extracted flower oils more truly represent natural perfume as originally present in flowers. (Sethi, 2001) [62-63].

Expression

It is physical process in which pressure is applied to squeeze the oil out of the material or juice from plant. This was usually achieved by a tincture press. This method is employed when essential oils are thermo sensitive. It is used for isolating essential oils from lemon and orange peels. In general, expression involves squeezing any plant material at great pressures to press out oils or other liquids. The process is carried out by hand-operated presses or crushes in isolated rural areas or by gigantic mechanical presses in industrial centers. (Stahl, 2005, Rahman, 1989) [65, 55].

Infusion

Infusions are prepared by soaking a drug in water for a specialized period of time. The process can be either hot or cold, depending upon the type of the ingredients present as decomposition may occur at higher temperatures. Infusions are generally prepared for immediate use, as preservatives are absent. In some cases preservatives like alcohol are used and the infusions concentrated by boiling.

Percolation (Exhaustive Extraction)

Percolation in usually one of the most widespread methods for plant extraction since it does not require much manipulation or time. It is a continuous process in which the saturated solvent is constantly being displaced by fresh menstrum. Normally, percolation is not used as a continuous method because sample is steeled in solvent in the percolator for 24hrs (for up to three times), and then the extracted materials are collected and pooled.

In general process of percolation, particularly in the manufacture of concentrated liquid extracts preparation, the following problems may arise:

a) If the active substances are thermo-labile, evaporation of large volume of dilute percolate, may result in partial loss of the active constituents
b) In the case of alcohol- water mixture, evaporation results in preferential vaporization of alcohol leaving behind an almost aqueous concentrate which may not be able to retain the extracted matter in solution and hence get precipitated. In such cases the modification in general process of percolation is required as given below,

Reserved Percolation

In this case the extraction is done through the general percolation procedure. At the last, the evaporation is done under reduced pressure in equipment like a Climbing evaporator to the consistency of a soft extract (semi solid) such that all the water is removed. This is then dissolved in the reserved portion which is strongly alcoholic and easily dissolves the evaporated portion with any risk of precipitation.

Decoction

Decoctions are prepared in a similar manner to that of infusions but the ingredients are boiled with that of water for a specified period of time or till a definite volume is attained. The term decoction is used when the preparation is prepared using hard plant parts like root, bark, wood etc. Decoctions are usually the method of choice when working with tougher and more fibrous plants, barks and roots (and which have water soluble chemicals). Depending on the type of plant material used, strong decoctions are prepared in two general ways. The first involves boiling the mixture longer. This is usually indicated when working with larger woody pieces of bark. Longer boiling time, up to 2 hours or more, is sometimes necessary to break down, soften, and extract the larger pieces. Alternatively, when smaller woody pieces are used yet a stronger remedy is wanted, the decoction is prepared as above (boiling 20 minutes), then it is allowed to sit/soak overnight before straining out the herb. When straining, again, make sure to press on the cut herb pieces in the strainer to get as much moisture/decoction out of the herb pieces. (Mukherjee, 2007)

Ultrasound Extraction

Extraction of intracellular compounds by cell-lysis is done by using ultrasound. When sound wave propagate in liquid media results in high-pressure and low-pressure cycle. The main effects of ultrasound extraction can be summarized as:

- To increase the permeability of the cell walls
- To produce cavitations i.e. the spontaneous formation of bubbles in a liquid below its boiling point resulting from strong dynamic stressing
- To increase mechanical stressing of the cells so called interface friction

Treatment with the ultrasound plays a major role e.g. decomposition of the alkaloids in jaborandi leaves is observed after 30 s ultrasound treatment on the laboratory scale at 20KHz but in the case of foxglove leaves the content of digitalis glycosides fell when an ultrasound output representing the optimum formation of hydrogen peroxide during the extraction.

Hot continuous extraction (Soxhlet extraction)

Soxhlet extraction method described by soxhlet in 1837.In this methods fat and oil from solid material is extracted by repeated washing with organic solvent under reflux. Organic solvent commonly used are hexane and petroleum ether. Disadvantage of this process are-polar lipid, long time involved large volumes of solvents, hazards of boiling solvents. (Rahman, 1989) [55].

Digestion

The in-gel digestion is part of the sample preparation for the mass spectrometric identification of proteins in course of proteomic analysis. The in-gel digestion primarily comprises the four steps destaining, reduction and alkylation (R&A) of the cysteines in the protein, proteolytic cleavage of the protein and extraction of the generated peptides. (Stahl, 2005) [65].

Destaining

Proteins which separated by ID or 2D PAGE are usually visualized by staining by staining with dyes like Coomassie Brilliant (CCB) or silver. (Stahl, 2005) [65].

Reduction and alkylation
The staining and destaining is often followed by the reduction and alkylation (r&a) of the cystines or cysteines potentially embodied in the protein. Hereby the disulfide bonds of the proteins are irreversibly broken up and the optimal unfolding of the tertiary structure is obtained. The reduction to the thiol is accomplished by the reaction with chemicals containing sulfhydryl or phosphine groups such as dithiothreitol (DTT) or tris-2-carboxyethylphosphine hydrochloride (TCEP). (Bhat *et al*., 2005, Heinrich *et al*., 2004) [8, 34].

Digestion
The protein is cut enzymatically into a limited number of shorter fragments. These fragments are called peptides and allow for the identification of the protein with their characteristic mass and pattern. The serine protease trypsin is the most common enzyme used in protein analytics. (Buchanan *et al.,* 2007, Colegate *et al.,* 2008) [12].

Extraction
After finishing the digestion, the peptides generated in this process have to be extracted from the gel matrix. This is accomplished by one or several extraction steps. Drawbacks for the in-gel digestion are the extended time need and the multiple processing steps making the method error-prone in respect to contaminations. (Colegate *et al.,* 2008)

Extraction by Electrical Energy
In this method, electrical energy is used in the form of an electric field to accelerate extraction and improve the yield of the extraction. Extraction of scopolamine from the seeds and capsules of Indian thorn apple has been reported by this process.

Vertical or Turbo Extraction
Vertical or turbo-extraction is used. Here the drug to be extracted is stirred in the menstrum with a high-speed mixer or homogenizer. The shredding and shearing forces break down the drug material to a particle size, which is smaller than that of the material when it is first put in the mixer. The cells become highly disintegrated. The diffusion of extractive substances through the cell membranes is largely replaced by washing out from the destroyed cellular tissues. These result in substantially faster establishment of the maceration equilibrium and hence in a considerable saving of time. (Mukherjee, 2002 & Mohammed, 2008) [48, 47].

Counter Current Extraction
Incontinuous countercurrent extraction, a moving solution, emulsion, suspension or solid mass is extracted by liquid phase flooring against it. In relative counter current extraction, on the other hand, only one phase (as a rule the extraction solvent) is in motion, the other phase (usually the solid) remains stationary. (Rahman, 1989) [55].

Miscellaneous Methods
Following are miscellaneous method used for extraction.

Expression
This method is use to obtain fixed oils from plant material. This involves disruption of the cellular structure by the application of pressure to the material and allows oil to flow out of the material. This method is frequently used for soya oil, sunflower oil and olive oil. The rupture in the cell kernel causes the elution of oil in this method. (Heinrich *et al*., 2004) [34].

Pervaporation
This method is currently being developed and its success will depend on the generation of new membranes which show selective binding for particular chemical groups. Hydrophilic membranes may be used to remove polar materials, including water from organic solvents and hydrophobic membranes can be used to remove organic compounds from an aqueous phase. This method has been used to remove aroma compounds from fruit juices. (Buchanan *et al*., 2007) [12].

Sublimation
In this process some substances, change from solid to gas or vice versa without passing through a liquid state on heating or cooling. This method can be used to obtain the substance from dried plant material or a dry crude extract. Caffeine of high purity can be obtained by this method from dry tea leaves.

Extraction of alkaloid, sesquiterpene lactone and cardiac glycoside, flavonoids, other polyphenols, sterols, saponins, carbohydrate
Solvent extraction is the most popular method of extraction. The main groups of compound to be considered are fixed oils, fats and waxes, volatile or essential oils, carotenoids, alkaloids, glycosides, aglycones, phenolic compounds, polysaccharides and proteins. Polarity and pH are two important factors. A general outline of the solvents that would be appropriate for extraction depends on above classes of compounds. The methods given in this section are general ones based on common properties of phyto chemicals. (Bohlin, 1998) [9].

Alkaloids
All alkaloids contain at least one nitrogen atom and the compound is basic. This means that salt formation can occur in the presence of acid. This fundamental property of alkaloids is used in their extraction and further clean-up. Two methods may be used for alkaloid extraction. One is to basify the plant material using diethylamine or ammonia and extract with an organic solvent. (Mukherjee, 2002) [48].

Carotenoids
Carotenoids are responsible for red, orange and yellow pigments observed in the plant and animal kingdoms. They are generally tetraterpenoid and can be divided into hydrocarbons and oxygenated forms known as xanthophylls. Hydrocarbon tetraterpenoid are less polar and can be extracted into petroleum ether. Xanthophylls are more polar therefore be extracted into ethanol or mixtures of ethanol and less polar solvents such as chloroform. (Rastogi *et al*., 1999) [56-57].

Glycosides
Glycosides are relatively polar in nature and its polarity depends on both number and type of sugar the structure of the aglycone. Most glycosides can be extracted with polar solvents such as acetone, ethanol, methanol, water or mixtures of these. However, cardiac glycosides have bulky steroidal aglycone, which shows appreciable solubility in chloroform. When extracting into water, and sometime enzymatic breakdown also possible. This will not occur if boiling water

is used or if significant proportions of alcohol or ammonium sulphate are added to the extract. In some cases, it may be the aglycone rather than the glycoside that is to be extracted, and this requires hydrolytic separation of the aglycone and sugar before or after extraction. (Mohammed, 2008) [47].

Phenolic Compounds

These can exist as free phenols or in glycosidic form. Due to the multiplicity of hydroxyl functions, phenols tend to be relatively polar and dissolve in aqueous alcohols. As they are weak acids, they may also be extracted or partitioned into aqueous alkali as phenolate salts. A problem encountered with phenolic compounds is that they can undergo extensive polymerization a reaction by the action of polyphenol oxidizes. This reaction will responsible for the development of brown coloration in damaged plant material when exposed to the air and in certain extracts. The polymerization reaction is catalyzed by acid. (Kalia, 2005) [38].

Proteins

Due to the presence of free carboxylic, amino and phenolic groups on amino acid side- chains in proteins, most can be ionized at high or low pH values. The pH at which no net charge is carried is known as the isoelectric point and this will vary with each protein depending on the constituent amino acids. At pH values above the isoelectric point, the protein carries a net negative charge, and the pH values below the isoelectric point, a net positive charge is carried. As a result of this, most proteins can be extracted with water, buffers, dilute acid or base or simple salt solutions. However, more lipophilic proteins require the use of 70-80% alcohol. Selective precipitation of groups of proteins in a crude protein extract can be achieved by gradual addition of acetone, ethanol or ammonium sulphate. (Bohlin, 1998) [9].

Polysaccharides

Polysaccharides are polymers of sugars or sugar derivatives. Generally, there are three types of sugar polymers - those that are completely water soluble, those that partially dissolve in water and swell to form gels and lastly, those that are water insoluble. Polysaccharides that totally or partially dissolve in water can be extracted using cold or warm water.

Volatile Oils

Volatile oils are the odorous principals found in various plant parts. Because they evaporate in air at ordinary temperatures, they are called volatile oils, ethereal oils or essential oils. (Mukherjee, 2002) [48].

Fractionation, Purification and Isolation

While different techniques have evolved for the extraction of the phytoconstituents so as to obtain crude extracts (complex mixtures), fractionmixtures) and isolated (pure) components from natural sources. These are usually then subjected to a number of further analytical investigations in order to obtain more information on the properties of their constituent substances. Broadly, the nature of further investigations after extraction of a crude drug is of three types:

1. Qualitative chemical analysis - determination of the nature of the constituents of a mixture or the structure of an isolated compound.
2. Quantitative chemical analysis - determination of the purity of an isolated substance or the concentration of a single substance or group of substances in a mixture
3. Bioassays - determination of the biological or pharmacological activity of substances and the dose range over which they exert their effects.

The amount of fraction available is possibly the most important factor in making decisions about its future treatment and analysis. Investigative methods can be either non-destructive or destructive. Non-destructive methods mean that the sample can be recovered and used for other tests. Some physico-chemical procedures, e.g. chemical tests, analytical chromatography, mass spectrometry and all biological testing procedures are destructive i.e. they ule up the sample and generally it cannot be recovered. Another important factor is that th6 different types of analysis require the sample to be present in different types of medium. lt is always desirable to supply the fractions as solids, which can be weighed accurately and reconstituted in the appropriate solvents. The solid forms are usually produced from solutions by evaporation under reduced pressure to minimize decomposition or, in the case of aqueous solutions, by freeze-drying, sometimes called lyophilization.

Separation/ Fractionation/ Isolation

1) Sublimation
2) Distillation
3) Evaporation
4) Fractional liberation
5) Fractional crystallization
6) Fractional distillation
7) Chromatography

Fractionation

All separation processes involve the division of a mixture into a number of discrete fractions. These fractional may be physically discrete fraction.In which successive partition into diethyl ether, chloroform and ethyl acetate will often afford in turn flavonones and flavonols, methoxylated flavonoids and flavonoid monoglycosides. Di- and polyglycosylated flavonoids will remain in the residual aqueous phase. Saponins are water soluble compounds, and the crude saponin fractions are water soluble compounds, and the crude saponin fractions may be obtained. The alkaloids are basic compounds may be extracted either into aqueous acid solution after removal of neutral impurities with an organic solvent, or by treating the groups wet plant material with an alkaline substance such as CaCO3 powder andextracting with diethyl ether after standing overnight. Specific literature should be consulted for specific plant extraction problems. (Colagate*et al*., 2008, Rastogi *et al.,* 1999) [56-57].

Sublimation

Sublimation is used for isolation of caffeine from tea, for its purification of materials present in a crude extract and for separation of camphor.

Distillation

Steam distillation is much used to isolate volatile oils and hydrocyanic acid from plant material. The TAS oven is used for steam distillation on a semi micro scale for the direct transfer of volatile material from a powder drug to thin layer plate.

Fractional liberation

Some compounds are separated by fractional liberation from a

mixture. A mixture of alkaloid salts in aqueous solution, when treated with aliquots of alkali, will give first the weakest base in the free salt followed by base liberation in ascending order of basicity. If the mixture is shaken with an organic solvent after each addition, then a fractionated series of base will be obtained. A similar procedure is used for organic acids soluble in water immiscible solvents. In this case, starting with a mixture of the acid salts, it is possible to fractionally liberate the acids by addition of mineral acids. Sodium salts of acids on treatment with dilute HCl yield free organic acids.

Fractional crystallization

The method is used on the difference in solubility of the components of a mixture in a particular solvent. Frequently, derivatives of the particular components are used (picrates of alkaloids, osazones of sugars).

Chromatography

Chromatography represents a group of techniques for separation of compounds of mixtures by their continuous distribution between two phases, one of which is moving past the other.

Paper Chromatography

Paper is used as stationary phase and mobile phase is used for separation of components.

Solvent Recycling

For both economical and ecological reasons the recovery of solvent is important. Use of single solvent is much better rather than azeotropic mixture which is difficult to separate into individual components. Complex distillation procedure is used for solvent recovery. An adequate care must be taken in disposing of solvents in a way that causes least damage to the environment. Most scientific establishments have standard procedures for the disposal of organic solvents. (Colegate *et al.*, 2008, Bohlin, 1998) [9].

Analytical and advanced methods for the extraction and identification of metabolite *in vitro*

Accelerated Solvent Extraction (ASE)

This technique is one of the most promising pressure liquid chromatography automated extraction process, successfully employed for various pollutants, pharmaceuticals and lipids (Fig. 1) (carabias *et al*., 2005 and Benthin *et al*., 1999).ASE is an innovative sample preparation technique that combines elevated temperature and pressures on liquid solvents to achieve a faster and more efficient removal of analytes from various matrices. In the ASE system, the extraction process is carried out at temperatures exceeding the boiling point of a solvent. The extraction of analytes from plant material is based on the analyte solubility, mass transfer effects and disruption of surface equilibria by using temperature and pressure simultaneously. In particular, pressure facilitates extractions from plant material in which the analytes have been trapped in matrix pores or in water-sealed pores or in air-bubble-sealed pores. ASE equipment represents a good solution in savings in time, solvents and laboratory costs. The ASE automated system may be suitable for the monitoring secondary metabolite production during the *in vitro* establishment because of the reduced solvent consumption (from five to two) and a good reproducibility (probably due to the minimal handly of sample).

Earlier studies on this technology evaluated a range of chemical structures (curcuminoids, saponins, flavonoids, polyphenols, terpenes) present in different vegetal matrixes such as roots, leaves, fruits, herbs and rhizomes (Chena *et al*., 2007) [22]. The essential oils can be extracted by official procedures using Clevenger apparatus (Pharmacopoeia), but ASE extractor using *n*-hexane or dichloromethane in turn is suitable to obtain the same characteristic constituents of aromatic mother plant (Choa *et al*., 2007). This technology has only recently been used also for the extraction of plant constituents such as antioxidants (Mendiola *et al*., 2008) [46]. At higher temperatures, although most phenolic antioxidants are stable, catechin and epicatechin are degradated. In extracting polyphenols such as catechin and epicatechin from tea and grape seed, it was found that among water, methanol, ethanol and ethyl acetate, the solvent methanol had the highest yield (Alonso *et al*., 2001) [2]. ASE was also used for the extraction of saponins, phloroglucinols and procyanidins. Bertoli *et al*. investigated the accelerated solvent extraction of chlorogenic acid, flavonoids, hypericin and hyperforin from different rigenarated lines of *Hypericum perforatum* hairy roots (Anand *et al*., 2005) [3].

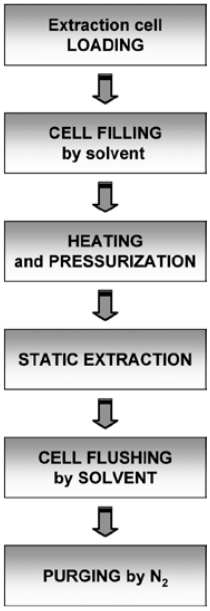

Fig 1: Operating mode by accelerated solvent extraction (ASE) technique.

Supercritical Fluid Extraction (SFE)

This technique uses the properties of gases above their critical points to extract selective soluble components from a plant material. Carbon dioxide is an ideal solvent for the extraction of natural products because it is nontoxic, nonexplosive, readily available and easy to remove from the extracted products (Fig. 2). Recently there has been an increasing interest in the SFE with carbon dioxide as solvent for the extraction of antioxidants from cultivated and *in vitro* plants (Cavero *et al*., 2006 & Caruso *et al*., 2000) [15].

SFE has the ability to use low temperatures leading to less deterioration of the thermally labile components in the extract. The main disadvantage of SFE is that it is often difficult to extract poly-oxigenated aglycons and their glycosides from plant matrices.

To enhance therecovery of these metabolites, it is important to find a proper organic cosolvent called 'modifier' such as methanol to add in suitable and small amounts to the plant material before the extraction. However, the recovery of the most polar analytes has not always satisfied. In the case of SFE extracts of *Rosmarinus officinalis*, the most active

antioxidant constituents are phenolic diterpenes such as carnosic acid, carnosol, rosmanol, epi-and iso-rosmanol together with rosmarinic acid (Senorans *et al*., 1996 & Carvalho *et al.*, 2005) [16]. On the other hand, rosmanol and epi-, iso-rosmanol are considered minor constituents resulting from the degradation of carnosic acid.There is a considerable interest in replacing the traditional methods with SFE technology, but the high costs of this equipment and the difficulties in the extraction of the more polar plant constituents have made it to be no so widespread yet. Nowadays, SFE technique is used especially to improve the quality of essential oils avoiding any thermal stress to the terpenes component (Reverchon *et al*., 1995).

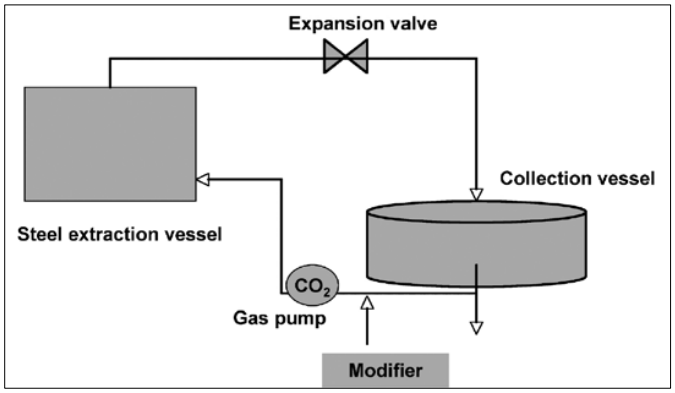

Fig 2: Scheme of supercritical fluid extraction (SFE) technique.

Microwave Assisted Extraction (MAE)

The use of MAE results in a significant reduction in the extraction time and solvent consumption in contrast to conventional liquid-solid extraction methods such as Soxhlet or Clevenger apparatus of different chemical classes of secondary metabolites (Carro *et al*., 1997 & Cavero *et al*., 2006). MAE was used also for the automatized extraction of important polar plant constituents such as is of lavones from soy or taxanes from Taxus. fast, sensitive and selective procedure employing a combination of microwave-assisted extraction and solid phase extraction was applied also to phenolic compounds in plant materials. However, MAE is expecially used for the laboratory scale extraction of essential oils and the microwave distillation is an alternative technique, which combines the microwave heating and the dry distillation at atmospheric pressure (Ferhata *et al*., 2006).

Solid Phase Micro Extraction (SPME)

The aroma of *in vitro* plant material can be studied by sampling directly the static or dynamic headspace by SPME technique (Fig. 3). Apolar and polar fibers, inserted in a special holder, are put in contact with the headspace developed over the PCTC sample to absorb the volatile compounds emitted spontaneously (Berrtoli *et al*., 2004 & Jain *et al*., 1991) [37]. As many stress factors influenced the volatile organic compounds emissions of plants in their habitat, producing plants under *in vitro* conditions offers important advantages in this respect as it implies exclusion of external contamination (Charron *et al*., 1995). [19] A reproducible *in vitro* growth involves control of important external factors such as temperature, light characteristic and relative humidity. Sudden changes in headspace composition can be explained and repeated sufficiently for statistical treatment under *in vitro* conditions (Mae *et al*., 2003) [42] Although *in vitro* plants can grow successfully when the culture container is properly sealed to avoid any external contamination, for the headspace analysis the conditions required are even more stringent in order to equilibrate the HS over plant sample before its adsorption. Furthermore, studies on the adsorptive characteristics of the culture medium, septa and/or the glassware were carried out in order to define potential interferences in the extraction yields (Mae *et al*., 2001) [43]. If the stabilizing time between fiber and headspace is too short, sensitivity is restricted; on the other hand, when it is increased too much, the total analysis time becomes excessively long. For practical reasons, a sampling time of 30 min was generally used as a compromise with respect to the equilibration requirements. Regarding the recovery of *in vitro* aroma by SPME, apolar monoterpenes together with some sesquiterpenes were studied. The headspace profile of *in vitro* plant material is sometimes really different from that obtained from the mother plant. Differences (up to 40%) in absolute amounts of each volatile component in *in vitro* plant samples can be caused by differences in the sample amounts and differences in the growth capacity. The dissimilarity in emission of mono- and sesquiterpenes between parent and *in vitro* plants suggests a different compartmentalization in the synthesis of monoterpenes (molecular formula: C10H16) compared with sesquiterpenes (molecular formula: C15H24). In addition, the SPME-GC-MS profiles of PCTC, generally showed compounds containing 6 carbon atoms which are originated from the degradation of the cell wall fatty acids.

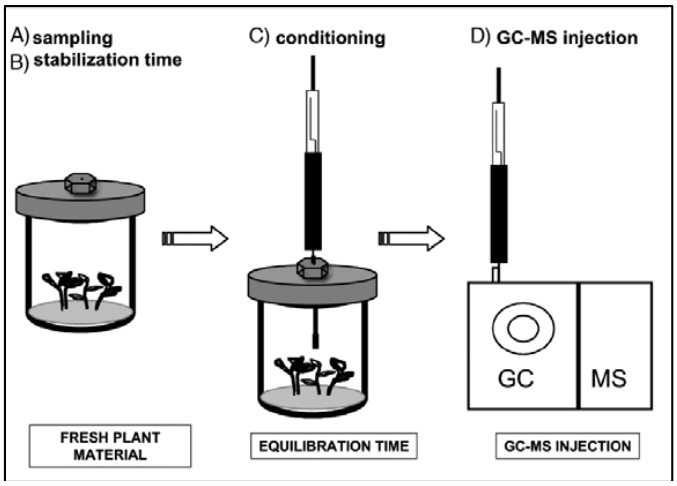

Fig 3: SPME-GC-MS analysis scheme for fresh plant material.

Chromatographic Separation and Detection of Phytochemicals

The wide chemical variety of plant constituents requires suitable quali-quantitative methods to evaluate one or more marker compounds after the extraction step (Fig. 4). It is generally accepted that a single analytical technique will not provide sufficient detection of the plant metabolomic profile which is generally a compromise between speed, selectivity and sensitivity of the analytical method (Exarchou *et al*., 2006).

Thin Layer Chromatography (TLC) and High Performance-Thin Layer Chromatography (HP-TLC)

TLC is the oldest analytical chromatographic technique for the screening of plant extracts. The separation process involves a suitable adsorbent (stationary phase) and a solvent or solvent mixture (mobile phase) (Satl. 1996). By TLC methods, a broad range of substances dissolved in all solvents even aggressive reagents can be tested. However, the separation efficiency of HPLC and capillary GC are

considerably higher than TLC. On the other hand, analytical TLC techniques require a very simple and low cost-equipment as well as reduced amounts of samples. The UV detection and densitometry also allow quantitative determination.Recently HP-TLC technique, allows faster and authomatic development (Potheir *et al*., 2001 & Galand *et al*., 2002). A preliminary phytochemical screening of PCTCs by thin layer chromatography is generally considered useful to perform quali-quantitative HPLC methods. A general inspection of the methanolic extracts obtained from cell suspension cultures, calli, regenerated shoots and roots of *Hypericum perforatum* was performed by analytical TLC (Pasqua *et al*., 2003). The ginsenoside content in different sources (field-grown roots, *in vitro* cultures, calluses, liquid cultures) of some ginseng species (*Panax ginseng, P. quinquefolium* and *P. vietnamensis*) were evaluated by HP-TLC technique combining an automatic TLC sampler and a scanner. In this study, HP-TLC was faster and simpler than HPLC (Kevers *et al*., 2004). The steviol-glycosides production in intact plants of *Stevia rebaudiana*was compared with different types of PCTCs by HP-TLC. For quantitative analysis, densitometric quantification of five glycosides (stevioside, rebaudiosides A, B and C and steviolbioside) was performed (Bondarew*et al*., 2001).

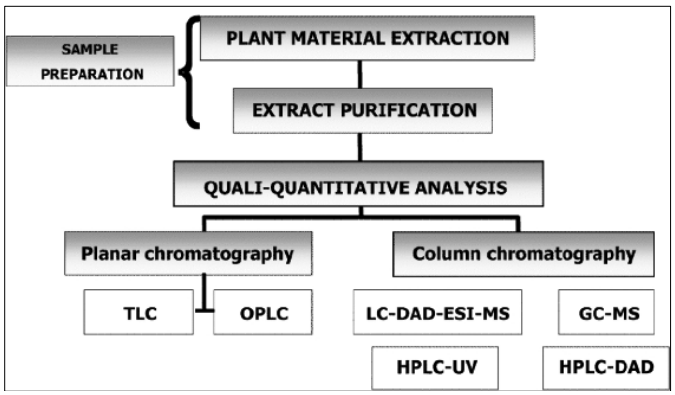

Fig 4: Flow-chart of analytical steps in the phytochemical investigation.

Over Pressured Layer Chromatography (OPLC)

It is relatively recent equipment, not yet generalized although it gives efficient separations in the analysis of essential oils and plant extracts (Botz *et al*., 2001). The OPLC technique is a unique liquid chromatography technology which made some authors even talk about 'flat columns'. In fact, OPLC system uses a programmable pump to deliver the mobile phase to the 'flat column' and the resulting forced flow leads to a faster separation and improved efficiency than capillary flow in the TLC system. OPLC is a planar chromatographic method which uses a pressured chamber where the vapor phase above the sorbent is practically eliminated. The eluent is pushed through the sorbent layer and a pump can perform the continuous development. OPLC technique like TLC can be used with various stationary phases. OPLC system which is described as a bridge between thin layer chromatography and high performance liquid chromatography is more rapid and reliable than TLC procedures for the analysis of a large number of complex plant extracts (Nyiredy, 2001) [50].

High-Performance Liquid Chromatography (HPLC)

It is a proven technique that has been used in laboratories worldwide over the past 30-plus years (Huie, 2001 & Luczkiewicz *et al*., 2005) [35]. Chromatograms of plant extracts are used as fingerprints and compared with standard compounds in order to identify the plant material and its constituents. HPLC is thus one of the best suited technique for an efficient separation of the crude plant extracts, as shown by Sakakibara (2003) who claim to have found a method capable of quantifying every polyphenol in vegetables, fruits and teas.The reversed-phase columns may be considered the most popular columns used in the analytical separation of plant secondary metabolites, even if new stationary phases have been exploited (Tanaka *et al*., 2002, 2004) [68-69]. The versatility of HPLC system was showed in the analysis of rosmarinic acid produced in hairy roots. An *in vitro* propagation protocol was developed to obtain shoot and root cultures from *Sanicula graveolens* (Apiaceae). Their content of chlorogenic acid and quercetin 3-O-glucoside was assessed by HPLC-diode array detector (HPLC-DAD) (Thiem, 2003) [71]. The research of plant material with phyto estrogenic activity was carried out also in PCTCs: the is of lavone accumulation *in vitro* cultures of *Genistatinctoria* and *Pueraria lobata*was used to investigate by HPLC-DAD. *In vitro* cultures of St. John's wort were found to contain hyperforin and three related polyprenylated acylphloroglucinol derivatives. The accumulation of these compounds after different stimulation was detected by HPLC-DAD system.

Changes in phenolic metabolism after elicitation with *Colletotrichum gloeosporioides*were monitored by HPLC-DAD in cell suspension cultures of *Hypericum perforatum*L. (Conceicao *et al*., 2006). Photodiode array detector is generally coupled with HPLC system as it allows to collect spectra in the established wavelength range and to define the spectral homogeneity (purity) of the analytes. This system is one of the most versatile tool in the screening of metabolic profiles of plant extracts. In addition, the analytical high performance liquid chromatography 'piloted' the preparative isolation of camptothecin and triterpenoids from *in vitro* cultures by the optimization of the experimental separations and checking the different fractions. However, the baseline separation using HPLC normally requires complex solvent gradient programs and long analysis times. In addition, unequivocal identification of flavonoids, one of the largest and widespread plant secondary metabolite classes, which have similar UV spectra and elution times, cannot be guaranteed. Furthermore, UV detection is unable to fulfill the phytochemical task, since lots of plant constituents have not chromophoric groups (Pasqua *et al*., 2004 & 2006).

Liquid Chromatography and Mass Spectrometry (LC-MS)

It combines the high separation power of high-performance liquid chromatography with the structural information of mass spectrometry. The sensitivity and specificity of LC-MS methods are drastically improved relatively to the traditional UV detection and allows the use of very fast chromatographic separations with high peak purity value (Tolstikow *et al*., 2002, Wolfender *et al*., 1998 & Halket *et al*., 2005) [33]. A key development of this technique is the interface Electron Spray Ionization that transfers analyte molecules from solution to the gas phase, suitable for mass analysis. The mass detection of a molecule is conditioned by the capacity of the analyte to ionize while being part of a complex mixture. Apart from the chemical properties of the molecule itself, eluent flow, composition of sample matrix as well as ionization source, influence ionization greatly. The use of ionization enhancers, sample clean-up methods and different ionization sources are

some of the possibilities that can improve ionization of analytes in the positive or negative modes.LC-MS mostly uses as soft-ionization sources the atmospheric pressure ionization, with the electrospray ionization or atmospheric pressure chemical ionization.

The performance of each soft-ionization mass spectrometer can be described by means of several intrinsic parameters: mass resolving power (or resolution), mass accuracy, linear dynamicrange and, sensitivity. Improvement of these parameters enables more effective identification of the molecular mass of the injected analyte. However, drawbacks are noise in the typical LC-MS raw data and retention time shifts which depend on the complexity of the matrix and ion suppression effects. For general applications in plant metabolomics, the isolation of one ion and the tandem mass spectrometry spectrometry experiments to obtained daughter fragments can be highly informative in order to elucidate metabolite structures directly in the non purified plant extract sample.

Based on these main features, LC-MS is at present a widely applied technique for the fast and sensitive quali-quantitative analysis of plant metabolites in order to compare wild, cultivated and *in vitro* plant material. Recent studies were carried out for the simultaneous analysis of salidroside and other main constituents in callus and plant extracts of *Rhodiola spp.* In these cases, the developed LC-MS method showed an extremely versatility for the simultaneous analysis of the transformation of cinnamyl alcohol precursor into rosavin or L-tyrosine as well as tyrosol precursors into salidroside in callus (Tolen *et al*., 2004 & Gyorgy *et al*., 2004) [32].

Ultra Performance Liquid Chromatography (UPLC)

It is an emerging technique for carrying out rapid and highly efficient quali-quantitative analysis. In fact, it makes possible to perform very high-resolution separations in short periods of time with little solvent consumption utilizing very small solid phase particles. Furthermore, the hyphenation of UPLC to mass spectrometry can be extremely advantageous for a very fast quali-quantitative analysis of complex plant matrices (Novakova *et al*., 2006 & Gruza *et al*., 2008) [49]. Robustness and reproducibility (retention time and mass accuracy) as well as efficient ionization of the analytes are essential for obtaining consistent data by UPLC-MS system. In conventional HPLC the choice of particle size must be a compromise as the smaller is the particle size, the higher column back-pressure is generating in system. On the contrast, the UPLC technology takes full advantage using columns packed with smaller particles and/or higher flow rates to perform superior resolution and sensitivity with shorter running time. UPLC removed the barrier of traditional chromatographic packing material by development of new, highly efficient, mechanically strong, 1.7 mm bridge hybrid particles that are stable over a broad pH operating range. Considering the shorter column wash-out time, UPLC methods can be regarded as "green" procedures because of the negligible solvent consumption for each chromatographic run. Due to very narrow and sharp peaks, more number of them may appear in less time which may facilitate in analysis of complex mixtures and that may give more qualitative information regarding plant metabolomic profile. Furthermore, the UPLC approach enables the detection of analytes at very low concentrations because of the improve signal-to-noise ratio. Quali-quantitative methods have been reported for the fast simultaneous determination of flavonoids and saponins in many plant extracts. This technology is quite new, but it has been applied also to the secondary metabolites produced in *in vitro* plant tissues. Some alkaloid fractions were analysed by UPLC in a study to describe the genetic engineering and expression of the terminal step of vindoline biosynthesis in *Catharanthus roseus* hairy root cultures (Magnotta *et al*., 2007) [44].

Gas-Chromatography Mass Spectrometry (GC-MS)

It is the most popular and useful analytical tool in the research field of volatile plant secondary metabolites (Admas, 1995). The advantages of GC clearly lie in its high sensitivity of detection for almost all the volatile chemical compounds present in the apolar plant extracts or essential oils. Furthermore, the high selectivity of capillary columns guarantees high resolution of many volatile compounds simultaneously within comparatively short times. This fact is extremely important in the case of essential oils, generally contain a hundred of terpenes and derivatives. In addition, the unambiguous identification of plant metabolite is possible by computer matching with commercial and experimental databases of mass spectra and retention indices. In fact, the method of ionization generally used in conjunction with gas chromatography is Electron Impact, a hard-ionization method which provides reproducible mass spectra and allows library searching. In the case of gas chromatography, it is well known that the volatility and thermal stability of the analytes represent sometimes a great limit.

Direct Infusion (DI)

The direct infusion using electrospray ionisation mass spectrometry (DI-ESI-MS), that is to say MS detection without prior chromatographic separation of the extract, is able to produce rich information on mass spectra of plant constituents (Mauri *et al*., 2000) [45].

For a preliminary qualitative screening, it is possible to inject directly into the mass spectrometer the unpurified plant extracts without the conventional chromatographic separation by liquid chromathography column. In fact, the 'molecular' ions in a complex sample may be sufficiently distinguished by their m/z values by ESI-MS direct infusion. Mass spectra are generally acquired in scan mode detection and ESI-MS conditions were optimized using the available standards for the different class of plant metabolites. However, it is important to point out that direct infusion technique has limited utility for the quantitative analysis of plant metabolites due to undesirable effects of ion suppression (Sterner *et al*., 2000) [66].

Direct infusion by ESI-MS has found recent application for the rapid characterization of vincristine and vinblastine in *Catharanthus roseus* as well as for the secondary metabolites in microbial extracts. The same technology was used to identify chemical differences that occurred in the expression of secondary metabolites by actinomycetes cultivated under six different fermentation conditions (Zahn *et al*., 2001) [79].

The reproducibility of ESI-MS experiments is generally guaranteed by the combinaton with chemometrics analysis (Cluster analysis, Principal Component Analysis). ESI-MS is considered competitive in terms of speed with other detection methods, especially in conjunction with MS/ MS analysis, but appropriate caution is recommended as it must be considered a potential valuable compromise between speed and information (Goodacrea *et al*., 2003) [30].

The described approach is specific, sensitive, rapid and does not require prepurification steps. It provides the fingerprint of

plant extracts, since it permits to detect simultaneously different constituents and it may be suggested for the discrimination of productive batches of *in vitro* plant material.

Conclusion

Natural product metabolite discovery program a long term capital-intensive program. Several extraction, fractionation/ separation and isolation methods are developed after which isolation of the active moiety and their chemical examination is performed. Microwave extraction, sonication, lyophilization, spray drying and vacuum drying have also been employed with good results. Generally the extraction is based on pharmacological activity rather than chemical nature of compounds. Further studies are carried out after the isolation of the active moiety to confirm the compound. The phytochemical investigation of a plant involves the selection, collection, identification and authentication, extraction of the plant material (first fractionation), fractionation/separation (second fractionation) and isolation (third fractionation) of the constituents, characterization of the isolated compounds and investigation of the biosynthetic pathways of particular compound, quantitative evaluations and pharmacological activities.

The extraction and identification of selected analytes by the classical analytical methods is generally a time and solvent—consuming process which no guarantees reproducible results. Therefore, modern extraction techniques such as ASE, MAE combined with chromatographic systems (OPLC, HPLC-DAD, GC-MS, LC-MS) or direct infusion (DI-ESI-MS) allow to develop rapid and effective analytical methods in order to define the quality and safety of metabolites extracted from plants.

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